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Optimal dietary alpha-linolenic acid/linoleic acid ratio improved digestive and absorptive capacities and target of rapamycin gene expression of juvenile grass carp (*Ctenopharyngodon idellus*)

Y.-Y. ZENG[1], W.-D. JIANG[1,2,3], Y. LIU[1,2,3], P. WU[1,2,3], J. ZHAO[1], J. JIANG[1,2,3], S.-Y. KUANG[4], L. TANG[4], W.-N. TANG[4], Y.-A. ZHANG[5], X.-Q. ZHOU[1,2,3] & L. FENG[1,2,3]

[1] *Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, China;* [2] *Fish Nutrition and Safety Production University Key Laboratory of Sichuan Province, Sichuan Agricultural University, Chengdu, China;* [3] *Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Sichuan Agricultural University, Chengdu, China;* [4] *Animal Nutrition Institute, Sichuan Academy of Animal Science, Chengdu, China;* [5] *Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China*

Abstract

Growth performance, digestive and absorptive capacities and target of rapamycin (TOR), ribosomal protein S6 kinase 1 (S6K1) and eIF4E-binding protein (4E-BP) gene expression in the hepatopancreas and intestine of juvenile grass carp (*Ctenopharyngodon idellus*) fed graded ratios of dietary alpha-linolenic acid/linoleic acid (ALA/LNA) (0.01, 0.34, 0.68, 1.03, 1.41, 1.76 and 2.15) for 60 days were investigated. The results showed that ALA/LNA ratio of 1.03 significantly improved (i) per cent weight gain (PWG) and feed efficiency, (ii) hepatopancreatic trypsin, chymotrypsin, lipase, amylase and intestinal creatine kinase (CK) activities, (iii) hepatopancreatic trypsinogen-2 and chymotrypsinogen mRNA levels. Meanwhile, fish fed with ALA/LNA ratio of 0.68 significantly enhanced, (iv) Na^+/K^+-ATPase and γ-glutamyl transpeptidase activities in whole intestine, and alkaline phosphatase activities in the proximal intestine (PI) and distal intestine, (v) amylase, intestinal Na^+/K^+-ATPase alpha-subunit isoform 1, Na^+/K^+-ATPase alpha-subunit isoform 8 and CK mRNA abundances, (vi) TOR and S6K1 gene expression in the hepatopancreas and intestine of juvenile grass carp. Based on the quadratic regression analysis of PWG, cholecystokinin and leptin contents in the PI, optimal dietary ALA/LNA ratio of juvenile grass carp (8.78–72.00 g) was estimated to be 1.08, 1.19 and 1.05, respectively.

KEY WORDS: absorption, alpha-linolenic acid, *Ctenopharyngodon idellus*, digestion, linoleic acid, TOR signalling pathway

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Correspondence: X.-Q. Zhou and L. Feng, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu 611130, China. E-mails: xqzhouqq@tom.com, zhouxq@sicau.edu.cn (XQZ) and fenglin@sicau.edu.cn (LF)

Introduction

Alpha-linolenic acid (ALA, 18:3n-3) and linoleic acid (LNA, 18:2n-6) as the important member of n-3 and n-6 fatty acids, respectively, have been considered as the essential fatty acids (EFAs) for many freshwater fish (Smith *et al.* 2004). Deficient ALA could result in gill tissue disintegration in turbot (*Scophthalmus maximus*) (Bell *et al.* 1985) and growth retardation in juvenile Murray cod (*Maccullochella peelii peelii*) (Francis *et al.* 2007). Meanwhile, insufficient LNA could lead to vertebral column curvature in juvenile grass carp (Takeuchi *et al.* 1991) and poor feed efficiency (FE) in rainbow trout (*Oncorhynchus mykiss*) (Thanuthong *et al.* 2011). The poor FE was related to the repressed digestive and absorptive capacities in fish (Refstie *et al.* 2000). However, no study has addressed the impacts of dietary ALA and LNA on the digestive and absorptive capacities in fish.

Fish digestion and absorption functions were mainly dependent on the activities of digestive enzymes (such as trypsin, chymotrypsin, lipase and amylase) and brush border enzymes [such as Na^+/K^+-ATPase alkaline phosphatase (AKP), γ-glutamyl transpeptidase (γ-GT) and creatine kinase (CK)] (Hakim *et al*. 2006). However, little information was available regarding the influence of dietary ALA and LNA on the digestive and brush border enzyme activities in fish. In murine enteroendocrine STC-1 cells, optimal level of ALA and LNA could upregulate cholecystokinin (CCK) secretion (Tanaka *et al*. 2008; Shah *et al*. 2012). Moreover, CCK could stimulate trypsin secretion, thereby enhance its activity in the hepatopancreas of Atlantic cod (*Gadus morhua*) (Tillner *et al*. 2013). These data indicated that dietary ALA and LNA may affect the digestive enzyme activities in fish. Meanwhile, fish intestinal brush border could secrete numerous enzymes to assimilate nutrients (Tengjaroenkul *et al*. 2002). Study in the intestinal Caco-2/15 cells revealed that cell secretion function was associated with the cell membrane structure integrity (Mailhot *et al*. 2010). Furthermore, ALA and LNA are vital fatty acids for maintaining the membrane structure integrity in Caco-2 cells (Beguin *et al*. 2013). These data indicated that dietary ALA and LNA may affect the digestive and brush border enzyme activities in fish, which is valuable for investigation.

The enzyme activities were found to be connected with their gene expression in fish (Tovar-Ramírez *et al*. 2010). Recent study from our laboratory indicated that the enhancement of glutathione peroxidase and copper/zinc superoxide dismutase activities is partly attributed to the upregulating of their gene expression in grass carp intestine (Deng *et al*. 2014). Moreover, nutritional factors could influence the digestive and brush border enzyme activities by modulating their gene transcriptions in fish. Study in Jian carp demonstrated that isoleucine could enhance the activities of chymotrypsin, lipase and γ-GT partly through elevating their gene transcriptional levels (Zhao *et al*. 2012b). However, whether dietary ALA and LNA could affect digestive and brush border enzyme activities by modulating their gene expression in fish is unknown. Itoh *et al*. (2003) reported that dietary ALA and LNA could stimulate insulin secretion in mouse pancreatic β cells. In addition, insulin could enhance Na^+/K^+-ATPase activity by boosting its gene expression in rat skeletal muscle (Galuska *et al*. 2009). Thus, it is reasonable to speculate that dietary ALA and LNA may improve digestive and brush border enzyme activities partly via elevating their gene transcriptions in fish, which is worthy of investigation. Furthermore, target of rapamycin (TOR) is a highly conserved serine–threonine kinase in mammals; ribosomal S6 kinase 1 (S6K1) and eIF4E−binding protein (4E−BP) are two important downstream targets of TOR signalling pathway (Shimobayashi & Hall 2014). Study in Hela cells reported that TOR could regulate RNA polymerase (pol) I gene expression (Mayer *et al*. 2004). Recently, our laboratory firstly cloned the cDNA of TOR (GenBank accession number FJ899680 and GenBank accession number JX854449) of Jian carp and grass carp, respectively. Besides, studies from our laboratory had shown that nutrients, such as arginine (Chen *et al*. 2012) and choline (Wu *et al*. 2011), could affect TOR and 4E-BP gene expression in the intestine of Jian carp. However, little information addressed the effects of dietary ALA and LNA on the gene transcriptions of TOR, S6K1 and 4E-BP in animal. In rat, ALA and LNA could increase serum leptin concentration (Korotkova *et al*. 2001). Meanwhile, leptin could elevate TOR expression in rat hypothalamic (Cota 2006). These data indicated that dietary ALA and LNA may regulate fish digestive and brush border enzyme gene transcriptions partly via TOR signalling pathway, which warrants further investigation.

Grass carp (*Ctenopharyngodon idellus*) as a typical herbivorous teleost is the second most-produced finfish species in world (FAO, 2012). The commercial culture of grass carp is highly relied on artificial diets, which is based on the complete information of the nutrient requirements for this species (Tang *et al*. 2013). Although the amount of EFA was vital for fish normal growth, study in some fish species had demonstrated that the proportion of ALA and LNA unbalanced contributed to growth retardation in fish as well (Francis *et al*. 2007; Blanchard *et al*. 2008). To date, only one study investigated the effects of ALA and LNA on the growth of juvenile grass carp (Takeuchi *et al*. 1991). However, that research merely designed three levels of ALA and LNA (0.0, 5.0 and 10.0 g kg^{-1}) with a constant ratio of ALA/LNA (1 : 1). Meanwhile, that research was conducted without applying regression models, suggesting that 10.0 g kg^{-1} ALA and 10.0 g kg^{-1} LNA might be satisfying the demands for juvenile grass carp. Pesti *et al*. (2009) reported that regression analysis is an efficiency method for estimating nutrients requirement in animal. In line with the above evidence, it is necessary to further evaluate the optimal ALA/LNA ratio for juvenile grass carp.

Therefore, this study preliminarily investigated the effects of dietary ALA/LNA ratios on the growth performance of juvenile grass carp. On this basis, we firstly studied the

impacts of dietary ratios of ALA/LNA on the digestive and brush border enzyme activities and their mRNA levels as well as signalling molecules' (TOR, S6K1 and 4E-BP) gene expression of juvenile grass carp, which could provide partial molecular mechanism for the effects of dietary ALA/LNA ratios on the growth performance of fish. In addition, dietary optimal ALA/LNA ratio of juvenile grass carp was also estimated, which may be used in formulating commercial feeds for the intensive culture of grass carp.

Materials and methods

Experimental diets and design

The formulation of the experimental diets is shown in Table 1. According to the method described by Turchini *et al.* (2013), seven isonitrogenous and isolipidic experimental diets varying only in the dietary lipid source were formulated. Meanwhile, the experiment diets contained 50.0 g kg^{-1} of lipid and 350.0 g kg^{-1} of protein, respectively, according to Ji *et al.* (2011) and NRC (2011). Fish meal (Pesquera Lota Protein Ltd., Lota, Chile), casein (Hulunbeier Sanyuan Milk Co., Ltd., Inner Mongolia, China) and gelatin (Rousselot Gelatin Co., Ltd., Guangdong, China) were used as the protein sources. According to Li *et al.* (2013), three different lipid sources such as linseed oil (Hunan Yama Biotechnology Co., Ltd., Hunan, China), safflower oil (Shanghai Yuan Tian Edible Agricultural Products Ltd., Shanghai, China) and coconut oil (Lvyuan natural flavour oil refinery, Jiangxi, China) were utilized to formulate the experiment diet containing varying ratios of ALA/LNA (0.00, 0.35, 0.70, 1.05, 1.40, 1.75 and 2.10), with a constant total C_{18} PUFA (ALA + LNA) content. Ethoxyquin was added as the antioxidant (González-Ortiz *et al.* 2013). According to the method of Otsuka *et al.* (2013), final ratios of dietary ALA/LNA of the seven experimental diets were measured to be 0.01, 0.34, 0.68, 1.03, 1.41, 1.76 and 2.15. All ingredients were mixed, pelleted and stored at −20 °C until use as described by Lee *et al.* (2011).

Table 1 Diet formulation and composition[1]

Ingredients	g kg^{-1}
Fish meal	30.0
Casein	280.0
Gelatin	75.0
DL-methionine (99%)	1.4
Vegetable oil premix[2]	50.0
Alpha-starch	240.0
Corn starch	215.5
Vitamin premix[3]	10.0
Mineral premix[4]	20.0
$Ca(H_2PO_4)_2$ (220 g kg^{-1})	22.6
Choline chloride (600 g kg^{-1})	5.0
Cellulose	50.0
Ethoxyquin (300 g kg^{-1})	0.5

[1] Crude protein and total lipids were measured to be 347.4 and 46.9 g kg^{-1}.

[2] Linseed oil and safflower oil were added to achieve different alpha-linolenic acid/linoleic acid ratios (0.00, 0.35, 0.70, 1.05, 1.40, 1.75 and 2.10). Each mixture was made isolipidic with the addition of coconut oil.

[3] Per kilogram of vitamin premix (g kg^{-1}): retinyl acetate (500 000 IU g^{-1}), 2.40 g; cholecalciferol (500 000 IU g^{-1}), 0.40 g; D, L-α-tocopherol acetate (500 g kg^{-1}), 12.55 g; menadione (230 g kg^{-1}), 0.80 g; cyanocobalamin (10 g kg^{-1}), 0.83 g; D-biotin (20 g kg^{-1}), 4.91 g; folic acid (960 g kg^{-1}), 0.40 g; thiamine nitrate (980 g kg^{-1}), 0.05 g; ascorhyl acetate (930 g kg^{-1}), 7.16 g; niacin (990 g kg^{-1}), 2.24 g; meso-inositol (990 g kg^{-1}), 19.39 g; calcium-D-pantothenate (980 g kg^{-1}) 2.89 g; riboflavin (800 g kg^{-1}), 0.55 g; pyridoxine hydrochloride (980 g kg^{-1}), 0.59 g. All ingredients were diluted with corn starch to 1 kg.

[4] Per kilogram of mineral premix (g kg^{-1}): $FeSO_4$ H_2O, 23.110 g; $CuSO_4$ $5H_2O$, 0.010 g; $ZnSO_4$ H_2O, 0.620 g; $MnSO_4$ H_2O, 1.640 g; KI, 0.070 g; $NaSeO_3$, 0.005 g; $MgSO_4$ H_2O, 60.530 g. All ingredients were diluted with corn starch to 1 kg.

Feeding management

The procedures used in this study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. Juvenile grass carp were obtained from Fisheries (Sichuan, China). At the commencement of the experiment, juvenile grass carp were acclimated to the experimental conditions for 4 weeks, according to Ji *et al.* (2011). Subsequently, a total of 1260 juvenile grass carp, with an average initial weight of 8.78 ± 0.03 g, were randomly distributed into 21 experimental cages (1.4 × 1.4 × 1.4 m), each of which was equipped with a 100-cm-diameter disc of 1-mm gauze in the bottom to collect the uneaten food, as described by Tang *et al.* (2013). Each cage was randomly assigned to one of three replicates of the seven dietary treatments, and fish were fed with the respective experimental diets to apparent satiation four times a day for 60 days, according to Gu *et al.* (2013). Thirty minutes after feeding, uneaten feed was collected, dried and weighed to calculate the feed intake (FI), as described by Lim & Lee (2009). During the experiment, water temperature was 26 ± 2 °C. The dissolved oxygen was not <6.0 mg L^{-1}, according to Ashton *et al.* (2013). The pH was 7.0 ± 0.5, and the experimental units were under natural light and dark cycle as described by Tang *et al.* (2013). At the initiation and termination of the

feeding trial, the fish in each cage were counted and weighted to determine the final body weight (FBW), PWG and FE according to Yun *et al.* (2011).

Sample collection and analysis

The procedures of sample collection were similar to those previously described in another study conducted in our laboratory (Feng *et al.* 2013). Prior to each sampling, the fish were starved for 12 h as described by Dong *et al.* (2013). Thirty fish from the same population before the experiment and six fish from each cage at the end of feeding trial were selected for a determination of initial and final carcass proximate composition. Meanwhile, the proximate compositions of feed and fish carcasses were analysed according to the method described by Le & Fotedar (2014). Another 15 fish from each cage were anaesthetized in benzocaine bath (50 mg L^{-1}) according to Zhao *et al.* (2012a), and then, the intestine and hepatopancreas were quickly removed, weighed and frozen in liquid nitrogen and then stored at −80 °C for later analysis as described by Liland *et al.* (2013).

The hepatopancreas and intestine samples were homogenized in 10 volumes (w/v) of ice-cold physiological saline and centrifuged at 6000 ***g*** for 20 min at 4 °C, and the supernatant was conserved for enzyme activity analysis, according to Heidarieh *et al.* (2013). Intestinal and hepatopancreatic protein contents were measured using the Coomassie Brilliant Blue dye binding technique. The measurements were obtained with an absorbance at 595 nm, and the protein content was calculated according to the standard curves (Bradford 1976). Trypsin and chymotrypsin activities were determined by the method of Hummel (1959). Lipase and amylase were assayed as described by Furné *et al.* (2005). Na^+/K^+-ATPase, AKP, γ-GT and CK were determined according to the procedure described by McCormick (1993), Bessey *et al.* (1946), Bauermeister *et al.* (1983), Tanzer & Gilvarg (1959), respectively. Leptin concentration was measured with an enzyme immunoassay kit using double-antibody sandwich method. The measurements were obtained at the absorbance of 450 nm according to the procedure described by Ganga *et al.* (2005).

Real-time PCR analysis

The procedures of RNA isolation, reverse transcription and quantitative real-time PCR were similar to those previously described in another study conducted in our laboratory (Wu *et al.* 2011). Total RNA was isolated from the hepatopancreas and all intestinal segments using RNAiso plus Kit (TaKaRa, Dalian, China). The quality and quantity of RNA was assessed by spectrophotometry at 260 and 280 nm and electrophoresis on 10 g kg^{-1} agarose gels as described by Qiao *et al.* (2013). Subsequently, cDNA was synthesized with 2 μL of total RNA using the PrimeScript™ RT reagent Kit (TaKaRa). For quantitative real-time PCR (qPCR), specific primers for the target genes were designed according to sequences of grass carp cloned in our laboratory. The primer sequences and optimal annealing temperatures are shown in Table 2. Real-time PCR was performed for these genes according to standard protocols. According to the results of our preliminary experiment concerning the evaluation of internal control genes (data not shown), β-actin was used as a reference gene to normalize cDNA loading. Target and housekeeping gene amplification efficiency were calculated according to the specific gene standard curves that were generated from 10-fold serial dilutions according to Luo *et al.* (2014). After verification that the primers amplified with an efficiency of approximately 100%, the results were analysed using the $2^{-\Delta\Delta C_T}$ method as described by Coccia *et al.* (2014).

Data analysis

Growth parameters were calculated using standard formulae, including survival rate (SR), PWG, FE, protein production value (PPV), lipid production value (LPV), ash production value (APV), HPC, hepatosomatic index (HSI), intestinal protein content (IPC) and intestosomatic index (ISI).

Results were presented as means ± standard deviation (SD). All data were subjected to a one-way analysis of variance (ANOVA). Differences among the treatment means were determined using a Duncan's multiple-range test at a $P < 0.05$ level of significance. Statistical analyses were performed using the SPSS Statistics 20.0 software as described by Harvey *et al.* (2014). A quadratic regression model was used to determine the optimal dietary ALA/LNA ratio, according to Zeitoun *et al.* (1976).

Results

Growth performance

Effects of dietary ALA/LNA ratios on growth parameters are given in Table 3. Survival rate was high in the

Table 2 Real-time primer sequences, thermocycling conditions and GenBank numbers

Gene	Sequences of primers	Thermocycling conditions	GenBank numbers
Trypsinogen-1			
Forward	5′-CTGCTGCTCACTGCTACAA-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	JX854450
Reverse	5′-TACTGCTCAGAACCCTCATT-3′	59.3 °C 30 s and 72 °C 30 s	
Trypsinogen-2			
Forward	5′-CAACTCCTCTTCGGTCATC-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	KM112096
Reverse	5′-GGAGCCCAAAGGCACATC-3′	59.3 °C 30 s and 72 °C 30 s	
Chymotrypsinogen			
Forward	5′-GGAAAGTCCATCATACACCC-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	JX854443
Reverse	5′-AGCCTCCAGCGAAGTTG-3′	60.4 °C 30 s and 72 °C 30 s	
Amylase			
Forward	5′-ACTATGTGCGTGGTAAGGT-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	FJ641975.1
Reverse	5′-CTTGATGTAATAGGCTCCC-3′	57.1 °C 30 s and 72 °C 30 s	
atp1a1a.1			
Forward	5′-TGCCATTGTAGCCGTAAC-3′	95 °C 30 s, 40 cycles of 95 °C 5 s,	JX854442
Reverse	5′-GGTGCCCAAAGGTAGAGG-3′	60.3 °C 30 s and 72 °C 30 s	
atp1a1a.4			
Forward	5′-GAGGTCGTTGCTGGTGAT-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	KM112094
Reverse	5′-CAGTGAGGGAAGAGTTGTC-3′	55.9 °C 30 s and 72 °C 30 s	
CK			
Forward	5′-CTCCTCGTTCACCCAGAC-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	JX854444
Reverse	5′-CAGCATCAAGGGATACGC-3′	61.4 °C 30 s and 72 °C 30 s	
TOR			
Forward	5′-TCCCACTTTCCACCAACT-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	JX854449
Reverse	5′-ACACCTCCACCTTCTCCA-3′	61.4 °C 30 s and 72 °C 30 s	
4E-BP			
Forward	5′-TTTCTACAAGCCAAGCCAC-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	JX854451
Reverse	5′-CAACCATGATGCCAAACC-3′	55.0 °C 30 s and 72 °C 30 s	
S6K1			
Forward	5′-TGGAGGAGGTAATGGACG-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	EF373673.1
Reverse	5′-ACATAAAGCAGCCTGACG-3′	59.4 °C 30 s and 72 °C 30 s	
β-Actin			
Forward	5′-GGCTGTGCTGTCCCTGTA-3′	95 °C 30 s, 40 cycles of 95 °C 5 s	M25013
Reverse	5′-GGGCATAACCCTCGTAGAT-3′	61.4 °C 30 s and 72 °C 30 s	

atp1a1a.1, Na^+/K^+-ATPase alpha-subunit isoform 1; *atp1a1a.4*, Na^+/K^+-ATPase alpha-subunit isoform 8; CK, creatine kinase; TOR, target of rapamycin; S6K1, ribosomal S6 kinase 1; 4E-BP, eIF4E-binding protein.

experiment, ranging from 96.7% to 100%, and showed no significant difference among the treatments ($P > 0.05$). FBW, PWG and LPV were significantly improved with increasing dietary ALA/LNA ratios up to 1.03 and depressed thereafter ($P < 0.05$). FI, FE, PPV and APV showed a similar trend with PWG, but no significant differences were noted for fish fed diets with ALA/LNA ratios of 0.68 and 1.03 ($P > 0.05$).

Growth and development of hepatopancreas and intestine

Hepatopancreas weight (HW), HSI, HPC, intestine length (IL), intestine weight (IW), ISI and IPC are presented in Table 4. HW, IL and IW of juvenile grass carp were significantly enhanced with increasing dietary ALA/LNA ratios up to 1.03 and then decreased ($P < 0.05$). HPC and IPC showed a similar tendency with HW ($P < 0.05$), but no significant difference was observed at the ratios of 0.68 and 1.03 ($P > 0.05$). However, when fish fed diets with ALA/LNA ratio of 2.15, HSI and ISI were higher than other treatments ($P < 0.05$).

Activities of digestive and brush border enzymes in the hepatopancreas and intestine

Influence of dietary ALA/LNA ratios on the activities of trypsin, chymotrypsin, lipase and amylase in the hepatopancreas and intestine are shown in Table 5. Trypsin and chymotrypsin activities in the hepatopancreas and intestine were significantly enhanced with increasing ALA/LNA ratios up to 1.03 and decreased subsequently ($P < 0.05$).

Table 3 Initial body weight (IBW, g fish^{-1}), final body weight (FBW, g), per cent weight gain (PWG), feed intake (FI, g fish^{-1}), feed efficiency (FE), survival rate (SR), protein production value (PPV), lipid production value (LPV) and ash production value (APV) of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days

	Dietary ALA/LNA ratios						
	0.01	0.34	0.68	1.03	1.41	1.76	2.15
IBW[1]	8.79 ± 0.03^{a}	8.77 ± 0.02^{a}	8.79 ± 0.04^{a}	8.77 ± 0.03^{a}	8.79 ± 0.04^{a}	8.76 ± 0.01^{a}	8.78 ± 0.01^{a}
FBW[1]	50.41 ± 0.42^{a}	58.04 ± 0.67^{b}	69.00 ± 0.66^{d}	72.00 ± 1.09^{e}	69.11 ± 1.58^{d}	61.09 ± 2.04^{c}	49.43 ± 1.03^{a}
PWG[1]	473.72 ± 5.90^{a}	561.56 ± 8.95^{b}	685.29 ± 4.46^{d}	720.77 ± 12.86^{e}	686.54 ± 20.07^{d}	597.59 ± 22.77^{c}	463.27 ± 11.11^{a}
FI[1]	64.40 ± 0.80^{a}	72.37 ± 0.51^{b}	81.32 ± 2.63^{d}	82.25 ± 1.87^{d}	81.76 ± 1.37^{d}	78.40 ± 0.49^{c}	72.91 ± 1.28^{b}
FE[1]	64.64 ± 0.69^{b}	68.08 ± 0.50^{c}	74.08 ± 2.00^{de}	76.91 ± 2.37^{e}	73.78 ± 0.78^{d}	66.75 ± 2.35^{bc}	55.77 ± 1.75^{a}
SR[1]	96.67 ± 3.33^{a}	98.89 ± 1.92^{a}	99.44 ± 0.96^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	98.89 ± 1.92^{a}	98.33 ± 1.67^{a}
PPV[2]	32.00 ± 1.17^{b}	34.32 ± 1.30^{c}	38.73 ± 0.87^{e}	39.83 ± 2.00^{e}	36.89 ± 0.64^{d}	32.72 ± 0.56^{b}	26.56 ± 0.78^{a}
LPV[2]	142.28 ± 7.98^{a}	180.45 ± 11.90^{b}	233.37 ± 8.88^{d}	272.75 ± 19.00^{e}	222.72 ± 11.00^{d}	201.68 ± 14.68^{c}	129.86 ± 5.71^{a}
APV[2]	61.38 ± 3.51^{b}	62.27 ± 3.65^{b}	67.79 ± 2.52^{c}	70.25 ± 3.06^{c}	67.34 ± 2.98^{c}	63.32 ± 1.04^{b}	55.26 ± 2.40^{a}

[1] Values are means ± SD of three replicate groups. Values within the same row with different superscripts are significantly different ($P < 0.05$).
[2] Values are means ± SD of three replicate groups, with six fish in each group. Values within the same row with different superscripts are significantly different ($P < 0.05$).
PWG = (g weight gain/g initial body weight) × 100.
FE = (g wet weight gain/g feed intake) × 100.
PPV = (g fish protein gain/g protein intake) × 100.
LPV = (g fish lipid gain/g lipid intake) × 100.
APV = (g fish ash gain/g ash intake) × 100.

Table 4 Hepatopancreas weight (HW, g fish^{-1}), intestinal weight (IW, g fish^{-1}), intestinal length (IL, cm fish^{-1}), hepatosomatic index (HSI), intestosomatic index (ISI), hepatopancreas protein content (HPC) and intestinal protein content (IPC) of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days

	Dietary ALA/LA ratio						
	0.01	0.34	0.68	1.03	1.41	1.76	2.15
Hepatopancreas							
HW[1]	0.84 ± 0.05^{a}	1.04 ± 0.03^{c}	1.22 ± 0.04^{d}	1.31 ± 0.04^{e}	1.21 ± 0.07^{d}	1.08 ± 0.06^{c}	0.93 ± 0.05^{b}
HSI[1]	1.70 ± 0.14^{a}	1.84 ± 0.09^{bc}	1.77 ± 0.08^{ab}	1.82 ± 0.09^{bc}	1.79 ± 0.17^{ab}	1.82 ± 0.15^{bc}	1.90 ± 0.15^{c}
HPC[2]	14.55 ± 0.97^{a}	15.54 ± 1.23^{ab}	16.31 ± 0.66^{bc}	17.57 ± 1.40^{c}	16.08 ± 1.09^{b}	15.68 ± 1.07^{ab}	14.89 ± 1.11^{ab}
Intestine							
IW[1]	1.24 ± 0.05^{a}	1.32 ± 0.07^{b}	1.51 ± 0.05^{c}	1.61 ± 0.04^{d}	1.48 ± 0.08^{c}	1.36 ± 0.06^{b}	1.27 ± 0.06^{a}
IL[1]	21.67 ± 1.63^{a}	21.98 ± 1.84^{a}	24.20 ± 1.60^{b}	26.23 ± 1.59^{c}	24.70 ± 2.06^{b}	24.37 ± 1.79^{b}	22.60 ± 1.58^{a}
ISI[1]	2.49 ± 0.21^{c}	2.34 ± 0.15^{b}	2.19 ± 0.09^{a}	2.23 ± 0.14^{ab}	2.18 ± 0.21^{a}	2.31 ± 0.19^{ab}	2.58 ± 0.19^{c}
IPC[2]	5.48 ± 0.43^{a}	6.98 ± 0.48^{c}	7.51 ± 0.57^{cd}	8.11 ± 0.47^{d}	7.37 ± 0.57^{c}	6.85 ± 0.60^{c}	6.18 ± 0.54^{b}

[1] Values are means ± SD of three replicate groups, with 15 fish in each group. Values within the same row with different superscripts are significantly different ($P < 0.05$).
[2] Values are means ± SD ($n = 6$). Values within the same row with different superscripts are significantly different ($P < 0.05$).
HPC = [hepatopancreatic protein (g)/wet hepatopancreas weight (g)] × 100.
HSI = [wet hepatopancreas weight (g)/wet body weight (g)] × 100.
IPC = [intestinal protein (g)/wet intestine weight (g)] × 100.
ISI = [wet intestine weight (g)/wet body weight (g)] × 100.

However, activities of trypsin and chymotrypsin in the intestine showed no significant difference when fish fed diets containing ALA/LNA ratios of 0.68 and 1.03 ($P > 0.05$). Lipase activity in the hepatopancreas was higher at the ratio of 1.03, whereas lipase activity in the intestine reached the peak when fish fed diets with ALA/LNA ratio of 0.68 ($P < 0.05$). Amylase activities in the hepatopancreas and intestine were higher for fish when ALA/LNA ratio was 1.03 ($P < 0.05$), whereas no significant difference was observed at the ratios of 0.68 and 1.03

Table 5 The activities of trypsin (U g^{-1} tissue), chymotrypsin (U g^{-1} tissue), lipase (U g^{-1} tissue) and amylase (U g^{-1} tissue) in the hepatopancreas and intestine and cholecystokinin (CCK) content (ng g^{-1}) in the proximal intestine of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days

	Dietary ALA/LNA ratios						
	0.01	0.34	0.68	1.03	1.41	1.76	2.15
Hepatopancreas							
Trypsin	1.01 ± 0.04^{a}	1.04 ± 0.05^{ab}	1.11 ± 0.03^{c}	1.19 ± 0.05^{d}	1.12 ± 0.01^{c}	1.11 ± 0.05^{c}	1.08 ± 0.04^{bc}
Chymotrypsin	28.11 ± 2.81^{a}	29.89 ± 2.02^{a}	46.40 ± 2.19^{c}	52.20 ± 4.40^{d}	43.72 ± 3.24^{c}	33.91 ± 3.24^{b}	30.34 ± 2.76^{a}
Lipase	715.11 ± 103.04^{a}	799.25 ± 103.04^{a}	1598.49 ± 130.34^{d}	1682.62 ± 130.34^{d}	1219.90 ± 103.04^{c}	1051.64 ± 103.04^{b}	967.51 ± 103.04^{b}
Amylase	874.85 ± 36.04^{a}	929.22 ± 29.38^{bc}	990.46 ± 13.28^{de}	1021.93 ± 34.31^{e}	957.23 ± 36.47^{cd}	915.13 ± 20.94^{b}	863.71 ± 21.16^{a}
Intestine							
Trypsin	0.64 ± 0.02^{a}	0.67 ± 0.02^{b}	0.69 ± 0.02^{c}	0.71 ± 0.02^{c}	0.71 ± 0.03^{c}	0.67 ± 0.03^{b}	0.65 ± 0.02^{ab}
Chymotrypsin	18.29 ± 1.09^{a}	29.45 ± 2.39^{b}	47.29 ± 4.03^{e}	49.97 ± 3.24^{e}	37.92 ± 2.63^{d}	33.02 ± 3.24^{c}	29.00 ± 2.63^{b}
Lipase	799.25 ± 103.04^{a}	799.25 ± 103.04^{a}	1472.29 ± 103.04^{c}	1430.23 ± 130.34^{c}	1051.64 ± 103.04^{b}	967.51 ± 103.04^{b}	799.25 ± 103.04^{a}
Amylase	779.61 ± 16.69^{a}	871.68 ± 32.81^{c}	948.98 ± 15.52^{d}	963.65 ± 21.47^{d}	895.41 ± 21.77^{c}	835.47 ± 22.80^{b}	762.58 ± 26.88^{a}
Proximal intestine							
CCK	12.88 ± 0.19^{a}	14.45 ± 0.58^{b}	17.50 ± 0.75^{d}	18.15 ± 0.64^{d}	18.08 ± 0.68^{d}	16.13 ± 0.64^{c}	14.78 ± 0.67^{b}

Values are means ± SD (n = 6). Values within the same row with different superscripts are significantly different ($P < 0.05$).

($P > 0.05$). Meanwhile, CCK showed a similar trend with amylase activities in the hepatopancreas and intestine ($P < 0.05$).

The brush border enzyme activities in the proximal intestine (PI), midintestine (MI) and distal intestine (DI) of juvenile grass carp fed diets with different dietary ALA/LNA ratios are presented in Table 6. The best AKP activity in the PI and DI was observed when fish fed diets with ALA/LNA ratio of 0.68 ($P < 0.05$). However, dietary ALA/LNA ratios did not have a significant effect on the AKP activity in the MI of juvenile grass carp ($P > 0.05$). Meanwhile, the activities of Na^+/K^+-ATPase in the PI and MI and γ-GT in three intestinal segments had a similar tendency with AKP activity in the PI, whereas the activities of Na^+/K^+-ATPase in the DI and CK in three intestinal segments were higher when fish fed with dietary ALA/LNA ratio of 1.03 ($P < 0.05$).

Digestive and brush border enzyme gene transcriptions in the hepatopancreas and intestine

As shown in Fig. 1, the mRNA levels of digestive enzymes (trypsinogen-1, trypsinogen-2, chymotrypsinogen and amylase) in the hepatopancreas of juvenile grass carp were affected by different dietary ALA/LNA ratios. Trypsinogen-1 mRNA level was significantly decreased with increasing dietary ALA/LNA ratios up to 1.03, and increased thereafter ($P < 0.05$). On the contrary, trypsinogen-2 mRNA level reached the peak at the ratio of 1.03 ($P < 0.05$), but showed no significant difference when ALA/LNA ratios were 1.03 and 1.41 ($P > 0.05$). Chymotrypsinogen mRNA level exhibited a similar tendency with trypsinogen-2 mRNA abundance ($P < 0.05$). However, amylase gene transcriptional abundance was significantly enhanced with increasing ALA/LNA ratios up to 0.68 and depressed subsequently ($P < 0.05$).

Na^+/K^+-ATPase alpha-subunit isoform 1 (*atp1a1a.1*) (a), Na^+/K^+-ATPase alpha-subunit isoform 8 (*atp1a1a.4*) (b) and CK (c) gene expression are exhibited in Fig. 2. The mRNA levels of *atp1a1a.1* in the PI and MI were higher when fish fed with ALA/LNA ratios of 0.68 ($P < 0.05$), but showed no significant difference when the ratios were 0.34, 0.68 and 1.03. In the DI, *atp1a1a.1* mRNA level was higher at the ratio of 1.03 ($P < 0.05$), but no significant difference was observed when fish fed diets containing ALA/LNA ratios of 0.68, 1.03 and 1.76 ($P > 0.05$). The transcriptional abundances of *atp1a1a.4* in the PI, MI and DI were higher when the ALA/LNA ratio was 0.68 ($P < 0.05$).

Table 6 The activities of alkaline phosphatase (AKP, mmol of nitrophenol released g^{-1} tissue h^{-1}), Na^+/K^+-ATPase (μmol of phosphorusreleased g^{-1} tissue h^{-1}), γ-glutamyl transpeptidase (γ-GT, mmol of 5-amino-2-nitrobenzoate released g^{-1} tissue min^{-1}) and creatine kinase (CK, μmol of phosphorus released g^{-1} tissue h^{-1}) in the proximal intestine (PI), midintestine (MI) and distal intestine (DI) of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days

	Dietary ALA/LNA ratios						
	0.01	0.34	0.68	1.03	1.41	1.76	2.15
AKP							
PI	73.38 ± 2.71^{a}	88.26 ± 5.66^{bc}	116.47 ± 2.81^{e}	110.70 ± 3.34^{d}	90.45 ± 3.43^{c}	84.33 ± 2.79^{b}	71.51 ± 3.29^{a}
MI	58.33 ± 1.90^{a}	59.03 ± 2.55^{a}	61.70 ± 2.57^{a}	61.60 ± 3.28^{a}	61.07 ± 2.30^{a}	60.17 ± 2.83^{a}	59.38 ± 2.46^{a}
DI	49.55 ± 2.56^{a}	50.86 ± 1.53^{ab}	53.58 ± 3.34^{b}	53.00 ± 2.30^{b}	52.40 ± 2.60^{ab}	51.81 ± 1.49^{ab}	50.74 ± 1.82^{ab}
Na^+/K^+-ATPase							
PI	67.17 ± 2.67^{a}	83.58 ± 3.44^{c}	93.04 ± 3.84^{e}	88.86 ± 2.82^{d}	82.86 ± 3.60^{c}	72.56 ± 2.60^{b}	64.16 ± 2.66^{a}
MI	31.85 ± 1.39^{a}	38.67 ± 1.69^{cd}	42.47 ± 2.10^{e}	40.22 ± 0.90^{d}	38.14 ± 0.68^{c}	37.34 ± 1.25^{c}	35.60 ± 1.32^{b}
DI	29.81 ± 2.54^{ab}	31.79 ± 2.28^{bc}	34.50 ± 3.22^{cd}	35.31 ± 3.12^{d}	32.40 ± 2.10^{bcd}	29.53 ± 2.89^{ab}	28.06 ± 2.16^{a}
γ-GT							
PI	23.53 ± 0.52^{a}	33.75 ± 0.76^{b}	54.90 ± 2.57^{g}	52.29 ± 1.40^{f}	50.07 ± 1.52^{e}	47.60 ± 2.05^{d}	43.39 ± 2.05^{c}
MI	62.98 ± 1.98^{a}	82.97 ± 3.26^{b}	132.44 ± 2.76^{f}	116.80 ± 2.38^{e}	103.50 ± 5.64^{d}	92.92 ± 1.33^{c}	85.59 ± 3.89^{b}
DI	35.34 ± 1.15^{a}	48.71 ± 2.25^{c}	65.12 ± 2.35^{f}	56.79 ± 1.76^{e}	53.58 ± 2.07^{d}	45.68 ± 1.41^{b}	44.08 ± 2.17^{b}
CK							
PI	76.42 ± 3.66^{a}	114.17 ± 7.16^{b}	160.75 ± 8.17^{e}	169.29 ± 6.65^{f}	146.27 ± 5.21^{d}	126.51 ± 6.19^{c}	107.63 ± 8.86^{b}
MI	63.64 ± 3.71^{a}	91.24 ± 7.34^{b}	114.27 ± 1.97^{d}	127.75 ± 9.05^{e}	119.54 ± 7.48^{d}	105.18 ± 3.32^{c}	94.52 ± 2.38^{b}
DI	61.61 ± 3.93^{a}	88.90 ± 3.37^{c}	110.81 ± 6.41^{e}	123.85 ± 6.38^{f}	107.16 ± 1.57^{e}	95.86 ± 3.72^{d}	82.53 ± 4.76^{b}

Values are means ± SD (n = 6). Values within the same row with different superscripts are significantly different ($P < 0.05$).

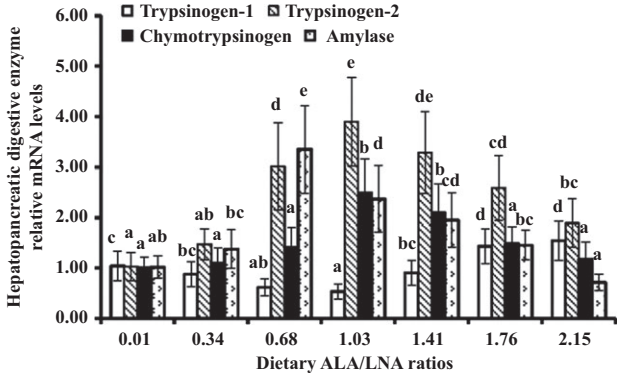

Figure 1 Relative mRNA levels of trypsinogen-1, trypsinogen-2, chymotrypsinogen and amylase genes in the hepatopancreas of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days. Values are means ± SD (n = 6). Different letters above a bar indicate statistically significant differences among treatments ($P < 0.05$).

However, *atp1a1a.4* mRNA levels in the MI and DI had no significant difference from the ratio of 0.34 to 1.41 ($P > 0.05$). The highest CK mRNA level in the PI was observed at the ratio of 0.68 ($P < 0.05$). Comparatively, CK mRNA level in the MI was significantly increased with improving dietary ALA/LNA ratios up to 1.03 and then plateaued ($P < 0.05$). When fish fed diets with ALA/LNA ratios of 0.68, 1.03 and 1.41, CK mRNA level in the DI was higher than other treatments ($P < 0.05$).

TOR, S6K1 and 4E-BP gene expression in the hepatopancreas and intestine

TOR, S6K1 and 4E-BP gene expression in the hepatopancreas are presented in Fig. 3. TOR and S6K1 mRNA levels were significantly elevated with increasing dietary ALA/LNA ratios up to 0.68 and decreased subsequently ($P < 0.05$). 4E-BP mRNA level showed an opposite tendency when compared with TOR mRNA abundance ($P < 0.05$). However, TOR, S6K1 and 4E-BP gene expression exhibited no significant difference when fish fed diets containing ALA/LNA ratios of 0.68 and 1.03 ($P > 0.05$).

In Fig. 4, TOR mRNA abundance in the PI was higher at the ratios from 0.34 to 1.41. In the MI and DI, TOR mRNA levels were significantly elevated with increasing dietary ALA/LNA ratios up to 0.68 and decreased thereafter ($P < 0.05$), whereas no significant difference was observed at the ratios of 0.68 and 1.03 ($P > 0.05$). S6K1 relative mRNA levels in three intestinal segments were significantly improved with increasing ALA/LNA ratios up to 0.68 and then decreased ($P < 0.05$). However, no significant

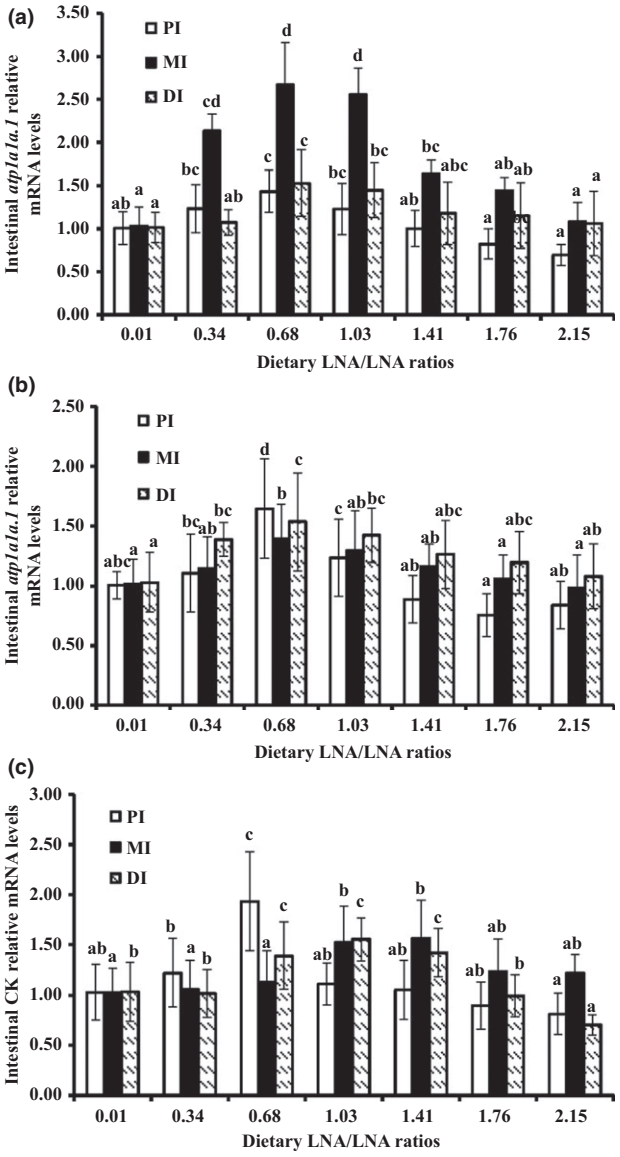

Figure 2 Relative mRNA levels of Na^+/K^+-ATPase alpha-subunit isoform 1 (*atp1a1a.1*) (a), Na^+/K^+-ATPase alpha-subunit isoform 8 (*atp1a1a.4*) (b), creatine kinase (CK) (c) genes in the proximal intestine (PI), midintestine (MI) and distal intestine (DI) of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days. Values are means ± SD ($n = 6$). Different letters above a bar indicate statistically significant differences among treatments ($P < 0.05$).

difference in the PI and MI was observed at the ratios of 0.68 and 1.03 ($P > 0.05$). 4E-BP gene expression had a similar tendency in three intestinal segments, and the lower mRNA levels were observed at the ratios of 0.68 and 1.03 ($P < 0.05$).

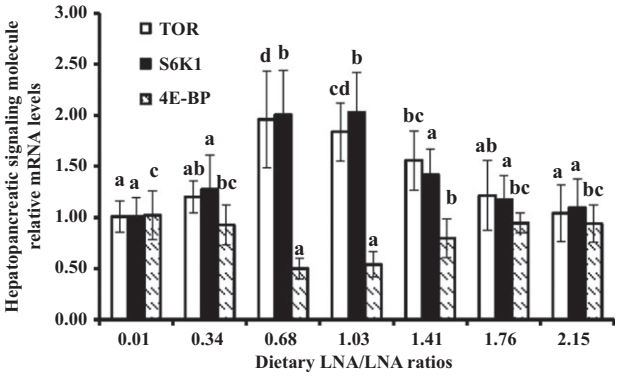

Figure 3 Relative mRNA levels of target of rapamycin (TOR), ribosomal S6 kinase 1 (S6K1) and eIF4E−binding protein (4E-BP) genes in the hepatopancreas of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days. Values are means ± SD ($n = 6$). Different letters above a bar indicate statistically significant differences among treatments ($P < 0.05$).

Leptin contents in the hepatopancreas and intestine

Leptin contents in the hepatopancreas and intestine are shown in Table 7. The highest leptin content in the hepatopancreas was observed at the ratio of 1.03 ($P < 0.05$), whereas no significant difference was observed when fish fed diets containing ALA/LNA ratios of 0.68 and 1.03 ($P > 0.05$). Leptin contents in three intestinal segments were significantly enhanced with elevating dietary ALA/LNA ratios up to 0.68 and decreased thereafter ($P < 0.05$). However, leptin content in the MI showed no significant difference when fish fed diets with ALA/LNA ratios of 0.68 and 1.03 ($P > 0.05$).

Quadratic regression analysis of PWG, CCK and leptin

Quadratic regression analysis of PWG is shown in Fig. 5a. The quadratic equations is $y = -217.82x^2 + 469.83x + 456.33$ ($R^2 = 0.9666$). CCK content in the PI is shown in Fig. 5b, and the quadratic equations is $y = -3.89x^2 + 9.2845x + 12.518$ ($R^2 = 0.8636$). Meanwhile, leptin content in the PI is shown in Fig. 5c. The quadratic equations is $y = -0.7027x^2 + 1.4763x + 0.7051$ ($R^2 = 0.8329$). Based on the quadratic regression analysis of PWG, CCK and leptin contents in the PI, optimal ALA/LNA ratio for maximum growth of juvenile grass carp (8.78–72.00 g) was estimated to be 1.08 (10.5 g kg^{-1} ALA + 9.7 g kg^{-1} LNA), 1.19 (11.1 g kg^{-1} ALA + 9.3 g kg^{-1} LNA) and 1.05 (10.4 g kg^{-1} ALA + 9.9 g kg^{-1} LNA), respectively.

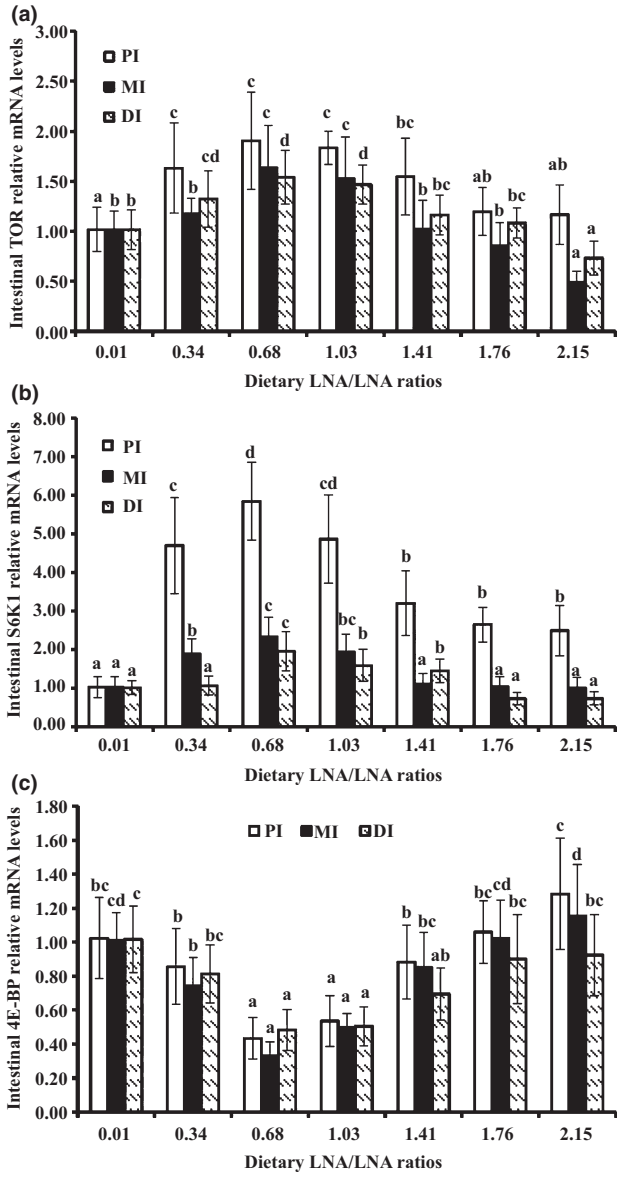

Figure 4 Relative mRNA levels of target of rapamycin (TOR) (a), ribosomal S6 kinase 1 (S6K 1) (b), eIF4E-binding protein (4E-BP) (c) genes in the proximal intestine (PI), midintestine (MI) and distal intestine (DI) of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days. Values are means ± SD ($n = 6$). Different letters above a bar indicate statistically significant differences among treatments ($P < 0.05$).

Discussion

Optimal ratio of ALA/LNA improved growth performance of juvenile fish

The present study exhibited the deficiency symptoms of juvenile grass carp in response to EFA limitation. Current study showed that the vertebral column of juvenile grass carp was curved when fish fed LNA-insufficient diet with ALA/LNA ratio of 2.15. This may be ascribed to the LNA metabolite, prostaglandins. Farina *et al.* (2011) reported that prostaglandins promoted intestinal calcium absorption, which could benefit the bone development in human. In addition, PWG, FI and FE of juvenile grass carp were significantly enhanced when fish fed with dietary ALA/LNA ratio of 1.03, which was lower than the results on juvenile yellow catfish (ALA/LNA: 1.17 and 2.12) (Tan *et al.* 2009) and Nile tilapia (ALA/LNA: 1.33) fry (El Husseiny *et al.* 2010). This discrepancy may be associated with fish species (Glencross 2009) and physiological age (Tocher 2010). Meanwhile, net nutrient depositions are accurate and important tools to study fish FE (Belal 2005). The present study showed that PPV, LPV and APV of juvenile grass carp were significantly increased when fish fed diets with proper ratio of ALA/LNA, suggesting that optimal ALA/LNA ratio could enhance nutrients deposition in juvenile fish. Moreover, nutrients deposition had close relations with digestive and absorptive capacities in Atlantic salmon (*Salmo salar* L.), which mainly relied on digestive and brush border enzyme activities (Nordrum *et al.* 2000; Chan & Horn 2004). Thus, we further investigated the effects of dietary ratios of ALA/LNA on the digestive and brush border enzyme activities in juvenile fish.

Optimal ratio of ALA/LNA increased the activities of digestive and brush border enzymes of juvenile fish

Digestive enzymes were synthesized within fish exocrine pancreas and ultimately secreted into the intestinal lumen (Gilloteaux *et al.* 2008). In the present research, optimal dietary ALA/LNA ratio significantly increased trypsin, chymotrypsin, lipase and amylase activities in the hepatopancreas and intestine of juvenile grass carp, indicating that proper ratio of ALA/LNA could improve the digestive capacities of juvenile fish. It may be attributed to the CCK. Murashita *et al.* (2009) reported that CCK was secreted in the PI and it played a key role in stimulating digestive enzymes secretion from the pancreas of Atlantic salmon. Thus, we further investigated the effects of dietary ALA/LNA ratios on CCK content in the PI of juvenile grass carp. In the present study, CCK content in the PI was significantly increased when fish fed diets containing optimal ALA/LNA ratio. Correlation analysis exhibited that the activities of trypsin, chymotrypsin, lipase and amylase in the intestine of juvenile grass carp had positive relation to CCK content, respectively

Table 7 Leptin concentration (μg L^{-1}) in the hepatopancreas (HP), proximal intestine (PI), midintestine (MI) and distal intestine (DI) of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days

	Dietary ALA/LNA ratios						
	0.01	0.34	0.68	1.03	1.41	1.76	2.15
Hepatopancreas							
Leptin	0.90 ± 0.06^{a}	1.04 ± 0.08^{b}	1.97 ± 0.17^{f}	2.05 ± 0.09^{f}	1.77 ± 0.14^{e}	1.61 ± 0.15^{d}	1.32 ± 0.12^{c}
Intestine							
Leptin in the PI	0.68 ± 0.05^{a}	1.08 ± 0.06^{b}	1.64 ± 0.11^{d}	1.33 ± 0.11^{c}	1.31 ± 0.08^{c}	1.16 ± 0.07^{b}	0.65 ± 0.06^{a}
Leptin in the MI	0.83 ± 0.05^{b}	0.99 ± 0.03^{c}	1.37 ± 0.13^{f}	1.31 ± 0.14^{ef}	1.26 ± 0.07^{e}	1.11 ± 0.06^{d}	0.68 ± 0.06^{a}
Leptin in the DI	0.42 ± 0.03^{a}	0.61 ± 0.05^{c}	1.38 ± 0.07^{f}	1.19 ± 0.06^{e}	1.17 ± 0.07^{e}	0.95 ± 0.06^{d}	0.54 ± 0.05^{a}

Values are means ± SD ($n = 6$). Values within the same row with different superscripts are significantly different ($P < 0.05$).

($r_{trypsin} = +0.950$, $P < 0.01$; $r_{chymotrypsin} = +0.922$, $P < 0.01$; $r_{lipase} = +0.814$, $P < 0.05$; $r_{amylase} = +0.818$, $P < 0.05$). These data implied that proper ratio of ALA/LNA increased digestive enzyme activities may be partly related to elevating the CCK content to enhance the secretion ability of pancreas in juvenile fish.

The brush border enzyme activities can directly reflect the absorptive capacity of fish (Mitra *et al*. 2008). Current study demonstrated that activities of Na^+/K^+-ATPase γ-GT and CK in three intestinal segments of juvenile grass carp significantly increased with optimal ratio of ALA/LNA, which noted that appropriate dietary ALA/LNA ratio could improve the absorptive capacity of juvenile fish. Interestingly, adequate ratio of ALA/LNA significantly increased AKP activities in the PI and DI except for MI of juvenile grass carp. The increased activity of AKP in the PI by optimal ALA/LNA ratio may be ascribed to the activation of peroxisome proliferator-activated receptor γ (PPAR γ). Yasui *et al*. (2005) reported that linolenic acid isomer, conjugated linolenic acid, could upregulate PPAR γ mRNA levels in Caco-2 cells. Lim *et al*. (2006) demonstrated that PPAR γ could promote CD 36 gene expression in smooth muscle cell of rat. Moreover, Nassir *et al*. (2007) showed that CD36 could facilitate long-chain fatty acid absorption in the PI of rat. Day *et al*. (1992) reported that long-chain fatty acid could enhance the AKP activities in human serum. These observations suggested that the increased AKP activity in the PI by optimal ALA/LNA ratio may be partly associated with activating PPAR γ transcription to enhance CD36 gene expression, thereby facilitating long-chain fatty acid absorption in the PI of juvenile fish, which needs additional investigation. On the other hand, proper ratio of ALA/LNA increased AKP activity in the DI may be owing to the short-chain fatty acid. Ringo *et al*. (2002) demonstrated that dietary ALA and LNA could benefit the gut microbiota growth in the DI of Arctic Charr (*Salvelinus alpinus* L.). Moreover, gut microbiota in the DI could convert the unassimilable fibre to short-chain fatty acid in the herbivorous fish (White *et al*. 2010). Furthermore, short-chain fatty acid could increase AKP activity in the human colon adenocarcinoma cells (Lecona *et al*. 2008). These data implied that optimal ALA/LNA ratio elevated AKP activity in the DI may be partly related to the enhancement growth of gut microbiota to promote the production of short-chain fatty acid levels in juvenile fish, which needs further investigation.

Fish digestive and brush border enzyme activities had close relation to the ontogenetic development of digestive organs (Feng *et al*. 2011). Current study revealed that dietary ALA/LNA ratio of 2.15 significantly enhanced HSI and ISI in juvenile grass carp, which may be ascribed to the lipid deposition. It was reported that higher ratio of ALA/LNA contributed to the lipid deposition in the liver of turbot (*S. maximus*) (Bell *et al*. 1995) and intestine of gilthead seabream (*Sparus aurata*) (Caballero *et al*. 2003), thereby increased digestive organs weight. Meanwhile, present study demonstrated that growth performance was significantly suppressed at the ratio of 2.15. These data implied that higher ratio of ALA/LNA increased HIS and ISI may be partly associated with the lipid deposition in the hepatopancreas and intestine of juvenile fish, which warrants further investigation.

From the above data, we can summarize that optimal ratio of ALA/LNA could inhibit the fatty deposition in the digestive organs and improve digestive and brush border enzyme activities in juvenile fish. Lambertucci *et al*. (2007) reported that the elevation of enzyme activities is partial attributed to the improvement of their gene transcriptional abundances. However, whether dietary ALA/LNA ratio influenced digestive and brush border enzyme activities via modulating their gene expression in fish is unknown. Therefore, we next investigated the impacts of dietary

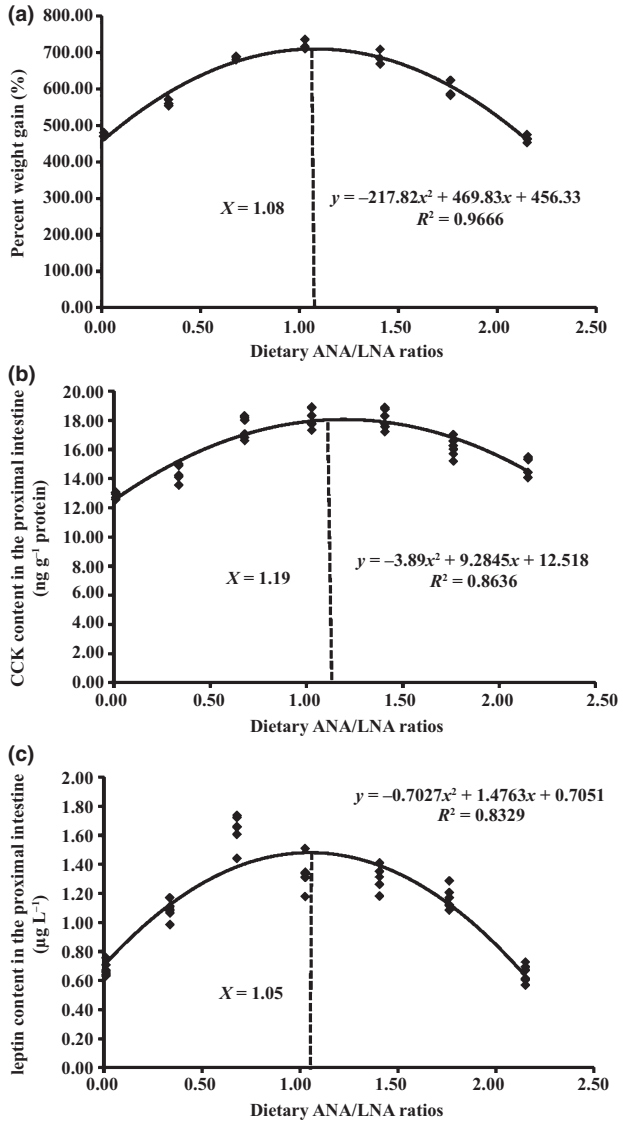

Figure 5 Quadratic regression analysis of per cent weight gain (a), cholecystokinin (CCK) (b) and leptin (c) content in the proximal intestine of juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with different alpha-linolenic acid/linoleic acid (ALA/LNA) ratios for 60 days.

ratios of ALA/LNA on the digestive and brush border enzyme gene transcriptions of juvenile fish.

Optimal ratio of ALA/LNA influenced the mRNA levels of digestive and brush border enzymes and TOR relevant signalling molecules in juvenile fish

The data reported herein demonstrated that appropriate ratio of ALA/LNA significantly upregulated chymotrypsinogen and amylase mRNA levels in the hepatopancreas of juvenile grass carp. Correlation analysis showed that chymotrypsin and amylase activities were positively related to their mRNA levels in the hepatopancreas of juvenile grass carp ($r_{\text{hepatopancreatic chymotrypsin}} = +0.867$, $P < 0.05$; $r_{\text{hepatopancreatic amylase}} = +0.874$, $P = 0.01$), indicating that optimal ratio of ALA/LNA elevated chymotrypsin and amylase activities may be partly ascribed to the enhancement of their gene expression. Moreover, trypsinogen-1 and trypsinogen-2 are important submits of trypsinogen in orange-spotted grouper (*Epinephelus coioides*) (Liu *et al*. 2013). Interestingly, current study demonstrated that proper ratio of ALA/LA significantly increased trypsinogen-2 mRNA levels, but decreased trypsinogen-1 gene expression in the hepatopancreas of juvenile grass carp. Optimal ratio of ALA/LNA increased trypsinogen-2 mRNA level and reduced trypsinogen-1 gene expression in the hepatopancreas of juvenile grass carp may be partly attributed to the CCK. Borgstrom *et al*. (1997) reported that CCK could promote the trypsinogen-2 mRNA levels and repress trypsinogen-1 mRNA abundances in rat pancreas. Meanwhile, data reported herein demonstrated that optimal ratio of ALA/LNA significantly increased CCK content in the PI of juvenile grass carp, implying that proper ratio of ALA/LNA upregulated trypsinogen-2 mRNA level and downregulated trypsinogen-1 mRNA abundance was partly associated with CCK in juvenile fish, which warrants further investigation. In addition, we also investigated the brush border enzyme gene transcriptions in the intestine of juvenile grass carp. Current study showed that optimal ratio of ALA/LNA significantly increased Na^+/K^+-ATPase alpha-subunit isoform 1 (*atp1a1a.1*), Na^+/K^+-ATPase alpha-subunit isoform 8 (*atp1a1a.4*) and CK mRNA levels in the intestine of juvenile grass carp. Correlation analysis exhibited that the activities of Na^+/K^+-ATPase in the intestine had positive correlation to the mRNA levels of *atp1a1a.1* ($r_{Na^+/K^+\text{-ATPase in PI}} = +0.886$, $P < 0.01$; $r_{Na^+/K^+\text{-ATPase in MI}} = +0.912$, $P < 0.01$; $r_{Na^+/K^+\text{-ATPase in DI}} = +0.856$, $P < 0.05$) and *atp1a1a.4* ($r_{Na^+/K^+\text{-ATPase in PI}} = +0.766$, $P < 0.05$; $r_{Na^+/K^+\text{-ATPase in MI}} = +0.885$, $P < 0.01$; $r_{Na^+/K^+\text{-ATPase in DI}} = +0.875$, $P = 0.01$); CK activities in the intestine were positive related to CK mRNA levels ($r_{\text{CK in MI}} = +0.793$, $P < 0.05$; $r_{\text{CK in DI}} = +0.774$, $P < 0.05$), indicating that optimal ratio of ALA/LNA increased brush border enzyme activities may be partly responsible for the enhancement of their mRNA levels in juvenile fish.

Several studies have demonstrated that TOR signalling pathway could regulate gene transcriptions of yeast (Mayer *et al*. 2004; Lee *et al*. 2009). In the present study, proper dietary ratio of ALA/LNA enhanced TOR and S6K1

mRNA levels and repressed 4E-BP gene expression in the hepatopancreas and intestine of juvenile grass carp. Further correlation analysis exhibited that mRNA abundances of trypsinogen-2 and amylase were positively related to TOR gene expression in the hepatopancreas of juvenile grass carp ($r_{\text{trypsinogen 2}} = +0.841$, $P < 0.05$; $r_{\text{amylase}} = +0.961$, $P < 0.01$). Likewise, *atp1a1a.1*, *atp1a1a.4* and CK mRNA abundances in the intestine of juvenile grass carp were positively correlated with the TOR gene expression ($r_{atp1a1a.1}$ in the PI $= +0.837$, $P < 0.05$; $r_{atp1a1a.1}$ in the MI $= +0.899$, $P < 0.01$; $r_{atp1a1a.1}$ in the DI $= +0.799$, $P < 0.05$; $r_{atp1a1a.4}$ in the PI $= +0.772$, $P < 0.05$; $r_{atp1a1a.4}$ in the MI $= +0.927$, $P < 0.01$; $r_{atp1a1a.1}$ in the DI $= +0.924$, $P < 0.01$; r_{CK} in the PI $= +0.722$, $P = 0.067$; r_{CK} in the DI $= +0.813$, $P < 0.05$). These data indicated that optimal ratio of ALA/LNA promoted digestive and brush border enzymes gene expression may be partly related to TOR of juvenile fish. However, little information is available regarding the effects of dietary ALA/LNA ratios on the gene expression of signalling molecules involved in the TOR signalling pathway of juvenile fish. We consider that optimal dietary ALA/LNA ratio enhanced TOR, S6K1 mRNA levels and reduced 4E-BP gene expression is likely attributed to the leptin in juvenile fish. Maya-Monteiro *et al.* (2008) reported that leptin could upregulate TOR and S6K1 expression and downregulate 4E-BP expression in macrophages of mice. Thus, we further investigated the effects of dietary ALA/LNA ratios on leptin contents in the digestive organs of juvenile fish. Data reported herein demonstrated that appropriate dietary ratio of ALA/LNA significantly increased leptin contents in the hepatopancreas and intestine of juvenile grass carp. Correlation analysis showed that TOR and S6K1 mRNA levels of juvenile grass carp were positively related to leptin contents in the hepatopancreas ($r_{\text{TOR}} = +0.886$, $P < 0.01$; $r_{\text{S6K1}} = +0.838$, $P < 0.05$) and intestine (r_{TOR} in the PI $= +0.859$, $P < 0.05$; r_{TOR} in the MI $= +0.810$, $P < 0.05$; r_{TOR} in the DI $= +0.715$, $P = 0.071$; r_{S6K1} in the PI $= +0.817$, $P < 0.05$; r_{S6K1} in the DI $= +0.792$, $P < 0.05$). Conversely, 4E-BP mRNA levels of juvenile grass carp were negatively related to leptin contents in the hepatopancreas ($r_{\text{4E-BP}} = -0.865$, $P < 0.05$) and intestine ($r_{\text{4E-BP}}$ in the PI $= -0.856$, $P < 0.05$; $r_{\text{4E-BP}}$ in the MI $= -0.820$, $P < 0.05$; $r_{\text{4E-BP}}$ in the DI $= -0.889$, $P < 0.01$). These data indicated that proper dietary ALA/LNA ratio enhanced digestive and brush border enzymes' gene expression in the hepatopancreas and intestine may be partly associated with elevating leptin contents in those tissues to modulate the TOR signalling pathway of juvenile fish.

Optimal dietary ALA/LNA ratio evaluated for juvenile grass carp

The above data clearly demonstrated that inadequate dietary ALA/LNA ratio could lead to negative effects on fish growth as well as decreasing digestive and absorptive capacities. In the present study, optimal dietary ALA/LNA ratio based on PWG, CCK and leptin in the PI was established to be 1.08, 1.19 and 1.05, respectively. All of the ratios in current study estimated based on different index were higher than the previous study conducted by Takeuchi *et al.* (1991) (ALA/LNA = 1:1), which could provide practical and useful information for commercial diet formulation.

Conclusion

In summary, four aspects of current study firstly showed that (i) optimal ALA/LNA ratio of 1.03 improved fish growth, which may be partly attributed to the enhancement of hepatopancreatic trypsin, chymotrypsin, lipase and amylase activities, and intestinal Na^+/K^+-ATPase, AKP, γ-GT and CK activities of fish. (ii) The increased activities of those enzymes by optimal dietary ALA/LNA ratio may be partly ascribed to the enhancement of their gene transcriptions. (iii) Proper ALA/LNA ratio of 0.68 elevated amylase mRNA levels in the hepatopancreas, and Na^+/K^+-ATPase alpha-subunit isoform 1 (*atp1a1a.1*), Na^+/K^+-ATPase alpha-subunit isoform 8 (*atp1a1a.4*) and CK mRNA abundances in the intestine may be partly ascribed to the TOR signalling pathway of fish. (iv) Optimal dietary ALA/LNA ratio of 1.03 elevated trypsinogen-2 mRNA abundance and reduced trypsinogen-1 gene expression may be partly responsible for CCK in the PI of fish. These results provided a partial molecular mechanism for the improvement of growth performance by optimal ALA/LNA ratio in fish. In addition, based on the quadratic regression analysis of PWG, CCK and leptin contents in the PI, optimal ALA/LNA ratio for maximum growth of juvenile grass carp (8.78–72.00 g) was estimated to be 1.08, 1.19 and 1.05, respectively.

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